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NOTE

MORE MOLECULAR EVIDENCE FOR INTERSPECIFIC RELATIONSHIPS IN *LIQUIDAMBAR* (HAMAMELIDACEAE)

JIANHUA LI AND MICHAEL J. DONOGHUE

Harvard University Herbaria, 22 Divinity Ave.,
Cambridge, MA 02138

There are four species of *Liquidambar* L. (Altingioideae, Hamamelidaceae). *Liquidambar orientalis* Miller occurs in western Asia and *L. styraciflua* L. in Northern and Central America, while *L. acalycina* Chang and *L. formosana* Hance are found in southeastern Asia (Bogle 1986). A phylogenetic analysis has recently been conducted based on DNA sequences of the plastid gene *mat*K (Li et al. 1997a). Li et al. found that the western Asian species *L. orientalis* is most closely related to the New World species *L. styraciflua*. This result is consistent with an earlier allozyme study (Hoey and Parks 1991). The finding is significant because it suggests a possible phytogeographical connection between the western Eurasian continent and the New World.

The close relationship of *Liquidambar styraciflua* and *L. orientalis* is moderately well supported by bootstrap values in the *mat*K analysis and no homoplasy was found. However, there are only seven phylogenetically informative characters in the data set. Therefore, it is desirable to gather more evidence to test this phylogenetic hypothesis. In this note, we report recent progress.

Three more regions of DNA have been sequenced, including the ITS (Internal Transcribed Spacer) region of nuclear ribosomal DNA (Baldwin et al. 1995), the intron of chloroplast gene *trn*L (Taberlet et al. 1991), and the exon 9-exon 12 region of the *GBSS* (Granule-Bound Starch Synthase) gene (Dai et al. 1996; Mason and Kellogg, unpubl. data).

DNA extraction, sequencing reactions, and PCR (Polymerase Chain Reaction) amplification for ITS were conducted as described in Li et al. (1997b, 1997c). PCR amplification of the *trn*L intron was carried out using primers c and d of Taberlet et al. (1991) with a thermocycler program of 30 cycles of 94°C for 35 sec., 55°C for 30 sec., and 72°C for 95 sec. The final cycle was

followed by a seven minute extension at 72°C. PCR amplification of the *GBSS* gene was conducted using primers *GBSS*F2 (5′TGGCATGGATACCCAAGAGT3′) and *GBSS*R2 (5′CCTTC-TTTCACAGTGTCAAC3′). We have successfully amplified a fragment of about 800 base pairs for several flowering plant taxa, including *Hamamelis* (Hamamelidaceae), *Stewartia* (Theaceae), and *Viburnum* (Adoxaceae). The thermocycler program consisted of a 60 sec. hotstart at 96°C and 40 three-temperature cycles, followed by a 15 minute extension at 72°C. Each cycle had denature and extension temperatures of 96°C for 60 sec. and 72°C for 90 sec., respectively. The annealing temperatures were 56°C, 54°C, and 52°C for the first two cycles, the second two, and the remaining 36 cycles, respectively. The annealing time was 60 sec. for all cycles. Sequencing reactions were carried out using Amersham cycle sequencing kit (Amersham Life Science Inc., Arlington Heights, IL) and following the manufacturer's instructions. Sequences were determined with an ABI 377 automated sequencer (Applied Biosystems, Inc. Foster City, CA). We obtained 348 base pairs from the ITS region (partial 5.8S plus ITS-2), 524 sites from the *trn*L intron, and 776 base pairs from the *GBSS* gene.

The parsimony analyses were conducted using the exhaustive tree search option of PAUP 3.1.1 (Swofford 1993). Trees were rooted using the same outgroup, *Mytilaria laosensis* Lecompte, as in the previous study (Li et al. 1997a), except that *Exbucklandia* R. W. Br., which is closely related to *Mytilaria* (Li et al. unpubl.), was used for the *GBSS* data set because we were unable to amplify the *GBSS* gene for *Mytilaria* due to its genomic DNA deterioration. Decay analysis (Donoghue et al. 1992) and 1000 bootstrap replicates (Felsenstein 1985) were carried out to indicate the relative support for the clades. Characters were unordered and unweighted, and gaps were treated as missing data.

The ITS data contained 82 variable sites, 15 of which were phylogenetically informative. The interspecific divergences between *Liquidambar* species ranged from 0.9–6.6%. A single most parsimonious tree was generated based on the ITS data set with a consistency index of 0.99. The tree was comprised of two clades, one of which contained *L. acalycina* and *L. formosana* and was supported by a bootstrap percentage of 100% and a decay index of nine steps. The other clade was composed of *L. orientalis* and *L. styraciflua* and was not strongly supported, with bootstrap and decay values of 69% and one step, respectively.

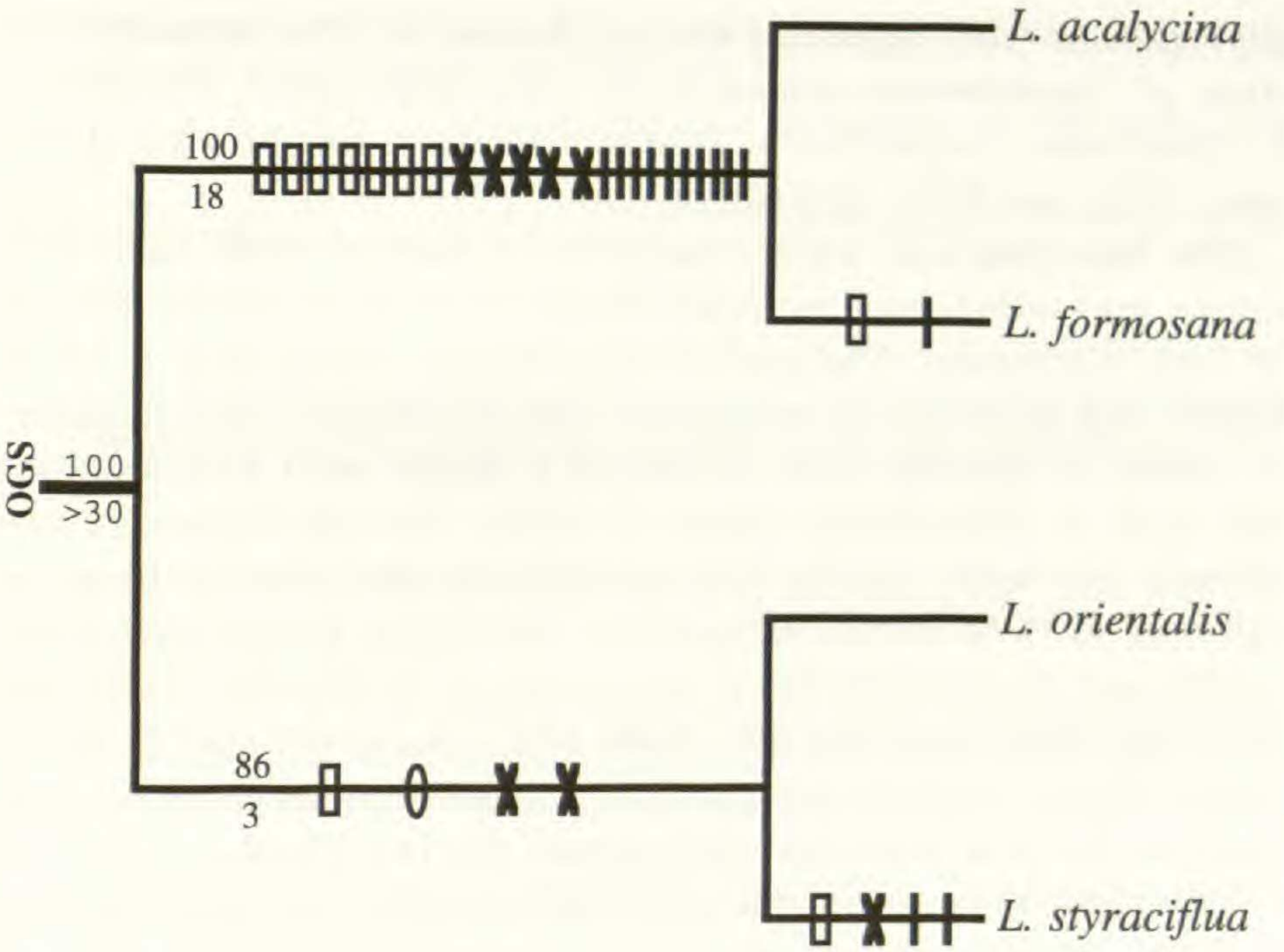

Figure 1. The single most parsimonious tree of 535 steps of *Liquidambar* based on sequences of ITS, *mat*K, *GBSS,* and *trn*L intron. CI = 0.98. Numbers above and below the branches are bootstrap percentages and decay index values, respectively. Symbols represent unambiguous, potentially informative changes of each data set along the branches.

The *trn*L intron data set had 21 variable sites, two of which were phylogenetically informative. The sequence divergences between *Liquidambar* species were from 0–0.8%. *Liquidambar acalycina* and *L. formosana* had identical *trn*L intron sequences. The parsimony analysis generated two equally short trees, one of which showed the tree topology produced by the ITS data, while the other tree did not resolve the relationships of *L. formosana, L. acalycina,* and the clade of *L. orientalis* and *L. styraciflua.* The consistency index was 1.0.

There were 105 variable sites in the *GBSS* data set, 15 of which were informative. Parsimony analysis resulted in one single shortest tree of 111 steps, with a consistency index of 0.96. In the

phylogenetic tree, eight and seven informative sites supported the clade of *Liquidambar orientalis–L. styraciflua* and *L. acalycina–L. formosana,* respectively. Bootstrap values for the two clades were 78% and 82%, respectively.

The four data sets were congruent, including *mat*K, ITS, *trn*L intron, and *GBSS,* and the combination of them created a data set of 3164 characters. The parsimony analysis, using both *Exbucklandia* and *Mytilaria* as outgroups with *Mytilaria GBSS* sequences coded as missing data, produced a single most parsimonious tree with a consistency index of 0.98. The phylogenetic tree showed the same species relationships as described in Li et al. (1997a). Both bootstrap percentages and decay values were high, 100% and 18 steps for the *L. acalycina–L. formosana* clade, and 86% and three steps for the clade of *L. orientalis* and *L. styraciflua.* Figure 1 shows the number of unambiguous changes from each of the four data sets that support the two clades.

This follow-up study strongly substantiates the previous hypothesis that the western Asian species *Liquidambar orientalis* is more closely related to the New World species *L. styraciflua* than to the southeast Asian species. Additionally, we conclude that sequences of the *GBSS* gene, especially the introns, provide another informative nuclear marker (besides nrDNA ITS) in resolving phylogenetic relationships among closely related species.

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